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/s/ Phillips

H. PHILLIPS

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PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(c).

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Docket Number	0434.74617	Type a plus sign (+) inside this box →	+

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TITLE OF THE INVENTION (280 characters max)

New Process for Continuous Purification and Concentration of Leucocytes

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ENCLOSED APPLICATION PARTS *(check all that apply)*

X	Specification	Number of Pages	13			Small Entity Statement	
X	Drawings	Number of Sheets	2			Other *(specify)*	

METHOD OF PAYMENT *(check one)*

	A check or money order is enclosed to cover the Provisional filing fee			
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

X NO

___ YES, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE Steven P. Schad DATE May 14, 1998

TYPED or PRINTED NAME Steven P. Schad REG. NO. *(if appropriate)* 32,550

___ Additional inventors are being named on separately number sheets attached hereto

PROVISIONAL APPLICATION FILING ONLY

Field of the invention

The present invention relates to the production of interferon in human leukocytes and specifically to a new process for continuous purification and concentration of leukocytes.

Prior art

Interferons constitute an endogenously produced immunologically active group of small proteins, which act as a natural defence against viral infections. They are synthesised and secreted by vertebrate cells following a virus infection. Interferons bind to the plasma membrane of other cells in the organism and induce an antiviral state in them by enhancing the production of three enzymes: an oligonucleotide synthetase, an endonuclease, and a kinase. In modern medical care, pharmaceutical compositions containing interferons are administered as a regimen against infections, specially viral infections, but also to generally boost the patient's immunological defence systems.

Interferons are presently manufactured via three different routes: recombinant, cell-line derived and human leukocyte derived. The human leukocyte derived interferon products can further be divided in partially purified and highly purified products. The present application concerns in particular highly purified human leukocyte interferon.

The large scale production of human leukocyte derived interferon is generally performed according to the process outlined by Kari Cantell **et al.** 1981 (Cantell, K., Hirvonen, S., Kauppinen, H-L. and Myllyla, G., Production of interferon in human leukocytes from normal donors with the use of Sendai virus, in Methods in Enzymology, vol 78, p. 29-38, and Cantell, K., Hirvonen, S. and Koistinen, V., Partial purification of human leucocyte interferon on a large scale, in Methods in Enzymology, vol 78, p.499-505.) The process according to Cantell can be summarised as follows: Pooled buffy coats from healthy donors are suspended in cold 0.83 % NH_4Cl and centrifuged. In this step the leukocytes are purified and separated from other blood cells. Approximately 30 % of the leukocytes are lost. The leukocytes are collected and incubated in modified Eagle's minimum essential medium (MEM). Further, the suspension is primed with priming interferon and then inoculated with Sendai virus, to initiate the production of interferon. The harvested crude interferon is then pooled and the interferon precipitated and purified further.

The leukocyte preparation steps are still mostly performed batch wise, using manually handled laboratory flasks and suitable equipment. Scale up has up to now been achieved by adding more flasks and centrifuges and naturally more personnel, handling these flasks. The production of interferon according to the state of the art is thus plagued by the drawbacks, typical for labour intensive processes: high labour costs, low reproducibility,

variations in yield etc. Nevertheless, most of the present processes have been focused on how to best utilise available laboratory equipment and methodology.

The present invention aims to overcome these drawbacks and to enable higher yield, better reproducibility and lower labour costs. Additionally, the invention aims to enable easier scale-up and GMP-verifiability of the process.

Summary of the invention

The present invention offers a solution to the above mentioned problems and shortcomings of conventional processes by introducing a process according to the attached claims. The inventive process has e.g. the advantages of being suitable for automation, thus improving the reproducibility, lowering the operator input needed and reducing labour costs. Further, the inventive process is easy to scale up and adapt to larger production volumes.

Short description of the drawings

The invention will now be described in greater detail below with reference to the accompanying drawings in which

Fig. 1 shows a block diagram showing the principles of a process according to the present invention;

Fig. 2 shows a schematic example of an automated leucocyte purification process according to the present invention.

Description of the invention

The present inventor has surprisingly shown that a continuous and automated process can be used in the purification and concentration of leukocytes. Said process comprises the following steps:

- plasma is removed from the leukocyte suspension by filtration
- NH_4Cl is added to the filtered leukocyte concentrate
- the mixture of leukocyte concentrate and NH_4Cl is lead through a static mixer
- the mixture of leukocyte concentrate and NH_4Cl is lead through a retention vessel
- the mixture of leukocyte concentrate and NH_4Cl is lead through a centrifuge
- the harvested cells are collected and processed further.

According to one embodiment of the invention, the filtration step for separation of plasma and leukocytes, is performed with the aid of hollow fibres, suitable for use in the inventive process and as a part of the inventive apparatus or system. The filtration parameters are balanced to produce, on one hand, a dense concentrated leukocyte fraction and, on the other hand, a pure plasma fraction, with as little discoloration as possible. The plasma fraction can be used as starting material in other processes since it is pure and contains no additives.

In order to achieve lysation of the erythrocytes in the leukocyte suspension, the concentrated suspension is mixed with an aqueous ammonium chloride solution (0.8 % w/v). The flow of ammonium chloride is preferably twice the flow of leukocytes. To ensure effective mixing, the mixture of leukocyte concentrate and the NH_4Cl solution is lead through a static mixer and further to a retention vessel. The retention vessel is designed so, considering the flow/volume ratio, that at retention time of about 10 minutes is achieved and that the solution becomes homogenous in the entire vessel.

Subsequently, the mixture of leukocyte concentrate and the NH_4Cl solution is supplied to a continuous centrifuge. The centrifuge can be a continuous or semi-continuous centrifuge of sanitary design and preferably a centrifuge adapted for sanitation without dismantling, Sanitation-In-Place (SIP) and also so called Clean-In-Place (CIP).

Further processing may include a second erythrocyte lysis step. Finally, the purified and concentrated leukocytes are transferred to a incubation vessel, where the interferon production is induced by adding Sendai virus. Preferably the interferon production is carried out in a bioreactor. The advantages with using a bioreactor as the incubation vessel is the possibility to achieve better control of the incubation step, easier scale-up, facilitated Sanitation-In-Place, and Cleaning-In-Place. Together with the other steps described earlier in the text, this will constitute a more complex process better adapted to industrial manufacturing of interferon.

Examples

Example 1. Pilot scale test run using pooled human buffy coats

Buffy coats are taken out from an ordinary production batch (pooled buffy coats from human donors) and emptied separately. Half of the amount of blood is processed through the inventive, experimental process and the other half according to an ordinary production process. In the experimental process, the plasma is separated by filtration through hollow fibres. The filtered plasma is fractionated and the fractions with highest absorbency at 280 nm (A280) are stored for later functional tests.

After the separation of plasma, the blood fraction flows directly into a mixing chamber, a static mixer, together with a flow of aqueous ammonium chloride solution (0.8 % w/v). The flow of ammonium chloride is preferably twice the blood flow, but it can vary in the range of 1,5 to 5 times said flow. The mixed solution then continuously enters the retention vessel. The retention vessel is designed, considering the flow/volume ratio in such a way that a retention time of about ten minutes is achieved, ensuring that the contents of the vessel become homogeneously mixed. This is the time needed for lysation according to

ordinary batch records. The lysate which exits the retention vessel is supplied directly into the semi-continuous centrifuge. The centrifuge is then intermittently harvested. The harvested cells are lysed once more and suspended with conventional medium to form a cell suspension with a high cell concentration. The cell suspension is then transferred to a bioreactor, containing a suitable medium and incubated for about 17 hours.

After about one and a half hour of the above 17 hours, Sendai virus is added to the bioreactor and the cells induced to produce interferon-alpha. After incubation, the interferon-alpha, which is secreted in the media, is recovered.

In example 1, the cell suspension is then added to 100 ml incubation flasks or to 3 litre laboratory bioreactors in order to compare cells from the ordinary production process and the cells from the experimental process. The interferon from the bioreactors and 100 ml flasks is then analysed for interferon content by an ELISA method specific for quantitative measurements of leukocyte derived interferon-alpha.

Table 1. Experimental yields: interferon

	Am unt add. (ml)	Cellconc. (x 107 st/ml)	ELISA (IU/ml)	Yield/cell (10-3 IU/cell)	Yield (%)	Yield/cell (%)	Tot. yield.
961009							
Exp.-cells	1,5	0,8	32000	4	100	100	100%
Ref.-cells	1,5	0,8	32000	4			
961023							
Exp.-cells	1,1	1	41000	4,1			
Exp.-cells	1,1	1	47000	4,7	85	90	155%
Ref.-cells	2	1	52000	5,2			
Ref.-cells	2	1,1	51000	4,6			
961030							
Exp.-cells	1	0,8	42000	5,3			
Exp.-cells	1	0,8	43000	5,4			
Ref.-cells	1	0,8	46000	5,8			
Ref.-cells	1	0,8	46000	5,8			
Exp.cells	1,5	1,1	61000	5,5			
Exp.-cells	1,5	1,1	54000	4,9	91	91	91%
Ref.-cells	1,5	1,1	65000	5,9			
Ref.-cells	1,5	1,1	64000	5,8			
961114							
Exp.- cells	1	1,1	38000	3,5			
Exp.- cells	1	1,1	37000	3,4			
Exp.- cells	1,5	1,7	56000	3,3			
Exp.- cells	1,5	1,7	56000	3,3	84	82	84%
Ref.-cells	1	1,1	42000	3,8			
Ref.-cells	1	1,1	46000	4,2			
Ref.-cells	1,5	1,6	70000	4,4			
Ref.-cells	1,5	1,6	64000	4			
961121							
Exp.- cells	1,5	1,1	58000	5,3			
Exp.- cells	1,5	1,1	53000	4,8			
Exp.- cells	2	1,5	73000	4,9			
Exp.- cells	2	1,5	66000	4,4	139	94	196%
Ref.-cells	2	0,7	29000	4,1			
Ref.-cells	2	0,7	46000	6,9			
Ref.-cells	3	1,1	56000	5,1			
Ref.-cells	3	1,1	51000	4,6			
961127							
Exp.- cells	1,1	1,1	41000	3,7			
Exp.- cells	1,1	1,1	47000	4,3			
Exp- cells	1,5	1,7	69000	4,1			
Exp.- cells	1,5	1,7	69000	4,1	128	92	193%
Ref.-cells	1,5	0,8	32000	4			
Ref.-cells	1,5	0,8	36000	4,5			
Ref.-cells	2,5	1,2	53000	4,4			
Ref.-cells	2,5	1,2	56000	4,7			
LabfermExp	45	0,8	46000	5,8			
LabfermRef	84	0,8	40000	5			

	Amount add. (ml)	Cellc nc. (x 107 st/ml)	ELISA (IU/ml)	Yield/cell .. (10-3 IU/cell)	Yield (%)	Yield/cell (%)	Tot. yield.
961204							
Exp.- cells	1	0,8	30000	3,8			
Exp.- cells	1	0,8	31000	3,9			
Exp.- cells	1,5	1,2	48000	4			
Exp.- cells	1,5	1,2	50000	4,2	87	96	87%
Ref.-cells	1	0,9	37000	4,1			
Ref.-cells	1	0,9	35000	3,9			
Ref.-cells	1,5	1,4	53000	4,4			
Ref.-cells	1,5	1,4	56000	4,1			
Labferm Exp	65	1,1	50000	4,5			
Labferm Ref	65	1,1	55000	5			
961206							
Exp.- cells	1,5	1	51000	5,1			
Exp.- cells	1,5	1	48000	4,8			
Exp.- cells	2,25	1,5	83000	5,5			
Exp.- cells	2,25	1,5	79000	5,3	116	116	78%
Ref.-cells	1	1	40000	4			
Ref.-cells	1	1	50000	5			
Ref.-cells	1,5	1,5	70000	4,7			
Ref.-cells	1,5	1,5	62000	4,1			
961211							
Exp.- cells	1,5	1,1	66000	6			
Exp.- cells	1,5	1,1	61000	5,5			
Exp.- cells	2	1,4	88000	6,3			
Exp.- cells	2	1,4	86000	6,1	106	89	106%
Ref.-cells	1,5	0,9	61000	6,8			
Ref.-cells	1,5	0,9	67000	7,4			
Ref.-cells	2	1,2	79000	6,6			
Ref.-cells	2	1,2	74000	6,2			
Labferm.Exp	65	1	68000	6,8			
Labferm.Ref	65	1	64000	6,4			
MEAN					**104**	**94**	**121**
STAND. DEV.					20	10	47
C.V.					19%	10%	39%
NO.					9	9	9

The results show a better yield with the inventive, experimental line. It is possible to get more cells from the experimental line in comparison with the conventional production line and thus a higher total yield of interferon since the yield per cell is about the same.

The functional tests for plasma separated by hollow fibres are performed through adding this plasma instead of the ordinary plasma to the medium when incubating leukocytes in small scale, 100 ml flasks. The interferon produced in these flasks was analysed by the ELISA-method and compared with ELISA-results from flasks incubated with ordinary plasma.

Table 2. Experimental yields: plasma quality

Plasma prep.	A 280	Amount add. (ml)	Media add. (ml)	Cellconc. (x 107 st/ml)	ELISA (IU/ml)	Yield/cell (10-3 IU/cell)	Yield/cell (%)	Yield (%)
M-5296, Ref.	25,6	1,6	40	1,2	63000	5,3	100	100
M-5296, Ref.	25,6	1,6	40	1,2	62000	5,2	100	100
961014	18,4	2,23	39,4	1,2	71000	5,9	115	115
961014	18,4	2,23	39,4	1,2	73000	6,1		
961028	17,3	2,38	39,2	1,2	64000	5,3	109	109
961028	17,3	2,38	39,2	1,2	72000	6,0		
961030	18,8	2,25	39,4	1,2	65000	5,4	102	102
961030	18,8	2,25	39,4	1,2	62000	5,2		
961114	14,6	2,81	38,8	1,2	66000	5,5	102	102
961114	14,6	2,81	38,8	1,2	62000	5,2		
961202	12,3	3,28	38,3	1,2	64000	5,3	106	106
961202	12,3	3,28	38,3	1,2	68000	5,7		
							107	**107**
MEAN							6	6
STAND. DEV.							5%	5%
C.V.							5	5
NO.								

Example 2. Sanitation trials to determine the results of Clean-In-Place (CIP)

Sanitation of the experimental process line comprising the centrifuge, plasma filter, static mixer and retention vessel was performed through flushing the syst m with NH4Cl directly after the blood except for the plasma filter which is flushed with PBS (phosphate buffered saline). The flow direction of PBS was reversed in the plasma filter. The whole system was then drained and thereafter flushed with PBS. The system was then drained again and filled with 0.5 M NaOH. The system was left to stand in 0.5 M NaOH for about one hour and then drained and refilled with the storage solution, 0.1 M NaOH.
Before use, the system is drained and the washed with PBS.

The system was disassembled at critical points and inspected visually to see that no cells, cell fragments etc. had been retained inside the system. Samples were taken from the wash solution before each trial when the wash solution had passed the whole system or as indicated in table 3. The samples were analysed for bacterial count (according to the European Pharmacopeia, 2nd Edition) and endotoxin determination (according to the European Pharmacopeia, 2nd Edition).

Table 3. Experimental results: sanitary status

	Bact. (cfu/ml)	Endotox. (EU/ml)
961009 Wash sol.:PBS	< 0.5	< 0.2
961023 Wash sol.:water	0,5	< 0.2
961030 Wash sol.:water	< 0,5	< 0,2
961114 Wash sol.:water, after hollow fibre Wash sol.:water, after retention vessel	< 0,5 1	< 0,2
961121 Wash sol.:water, filtrate Wash sol.:water, after retention vessel	< 0,5 < 0,5	ND
961127 Wash sol.:water	< 0.5	< 0,2
961204 Wash sol.:water	< 0,5	ND
961206 Wash sol.:water	< 0,5	ND
961211 Wash sol.:water	< 0,5	ND

No cells, cell fragments etc. was seen inside the system. Table 3 shows that the inventive system can be run under aseptic conditions and is suitable for CIP, that is cleaned in place.

Although the invention has been described with regard to its preferred embodiments, which constitute the best mode presently known to the inventors, it should be understood that various changes and modifications as would be obvious to one having the ordinary skill in this art may be made without departing from the scope of the invention which is set forth in the claims appended hereto.

Claims

1. Process for the continuous purification and concentration of leukocytes from human blood, **characterized** in that said process comprises the following steps:

- plasma is removed from the leukocyte suspension by filtration
- NH_4Cl is added to the filtered leukocyte concentrate in the form of an aqueous ammonium chloride solution
- the mixture of leukocyte concentrate and the NH_4Cl solution is lead through a mixing device
- the mixture of leukocyte concentrate and NH_4Cl solution is lead through a retention vessel
- the mixture of leukocyte concentrate and NH_4Cl solution is lead through a centrifuge
- the separated and harvested cells are collected and processed further.

2. Process according to claim 1, **characterized** in that the filtration is performed by leading the leukocyte suspension past a membrane filter with a pore size in the interval of 0.1 - 1.0 µm.

3. Process according to claim 1, **characterized** in that the retention vessel is designed in a way resulting in a retention time for the mixture of leukocyte concentrate and NH_4Cl of about 10 minutes.

4. Process according to claim 1, **characterized** in that the plasma removed in step (a) is recovered.

5. Process according to claim 1, **characterized** in that the process is automatically operated and adapted for CIP-cleaning and sanitation.

6. Process according to claim 1, **characterized** in that the harvested cells are subjected to a second lysis.

7. Process according to claim 1, **characterized** in that the harvested cells are incubated in a bioreactor.

8. Apparatus for continuous purification and concentration of leukocytes, **characterized** in that said apparatus includes the following devices:

- a membrane filter
- a static mixer
- a retention vessel
- a centrifuge.

9. Apparatus according to claim 8, **characterized** in that said apparatus is equipped for cleaning and sanitation, which cleaning and sanitation does not require the dismantling of the equipment, so called CIP and SIP.

10. Apparatus according to claim 8, **characterized** in that the membrane filter is a filter with a pore size in the interval of 0.4 - 0.6 μm.

11. Apparatus according to claim 8, **characterized** in that the retention vessel is designed in a way resulting in a retention time for a homogenous mixture of leukocyte concentrate and NH_4Cl of about 10 minutes.

12. Apparatus according to claim 8, **characterized** in that the centrifuge is adapted to continuous or semi-continuous harvest of cells.

Summary

Human leukocytes can be purified and concentrated using a continuous process, resulting in high yield and better reproducibility than the conventional batch processes. The total process economy is improved by minimizing the labour input, and increasing the productivity. The inventive process further has the benefits of allowing ease of automation and scale-up (Fig. 1).

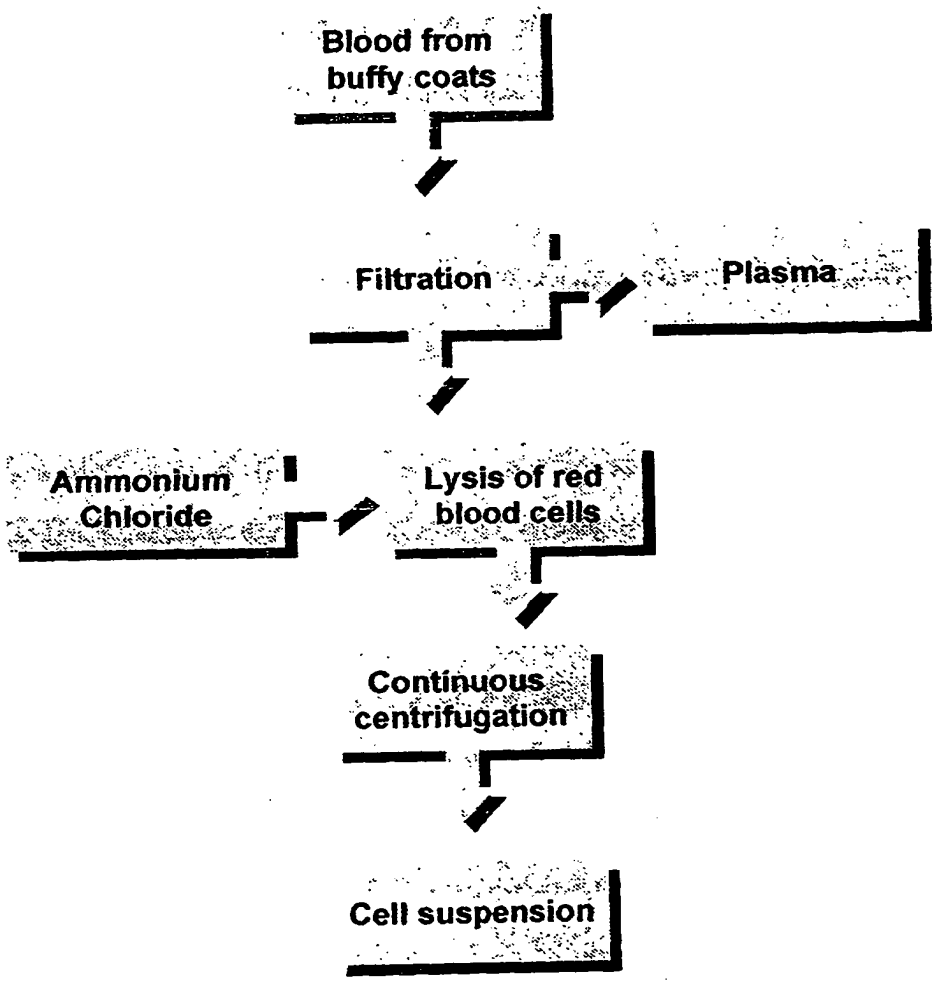
Blood from buffy coats
Filtration
Plasma
Ammonium Chloride
Lysis of red blood cells
Continuous centrifugation
Cell suspension

Fig. 1

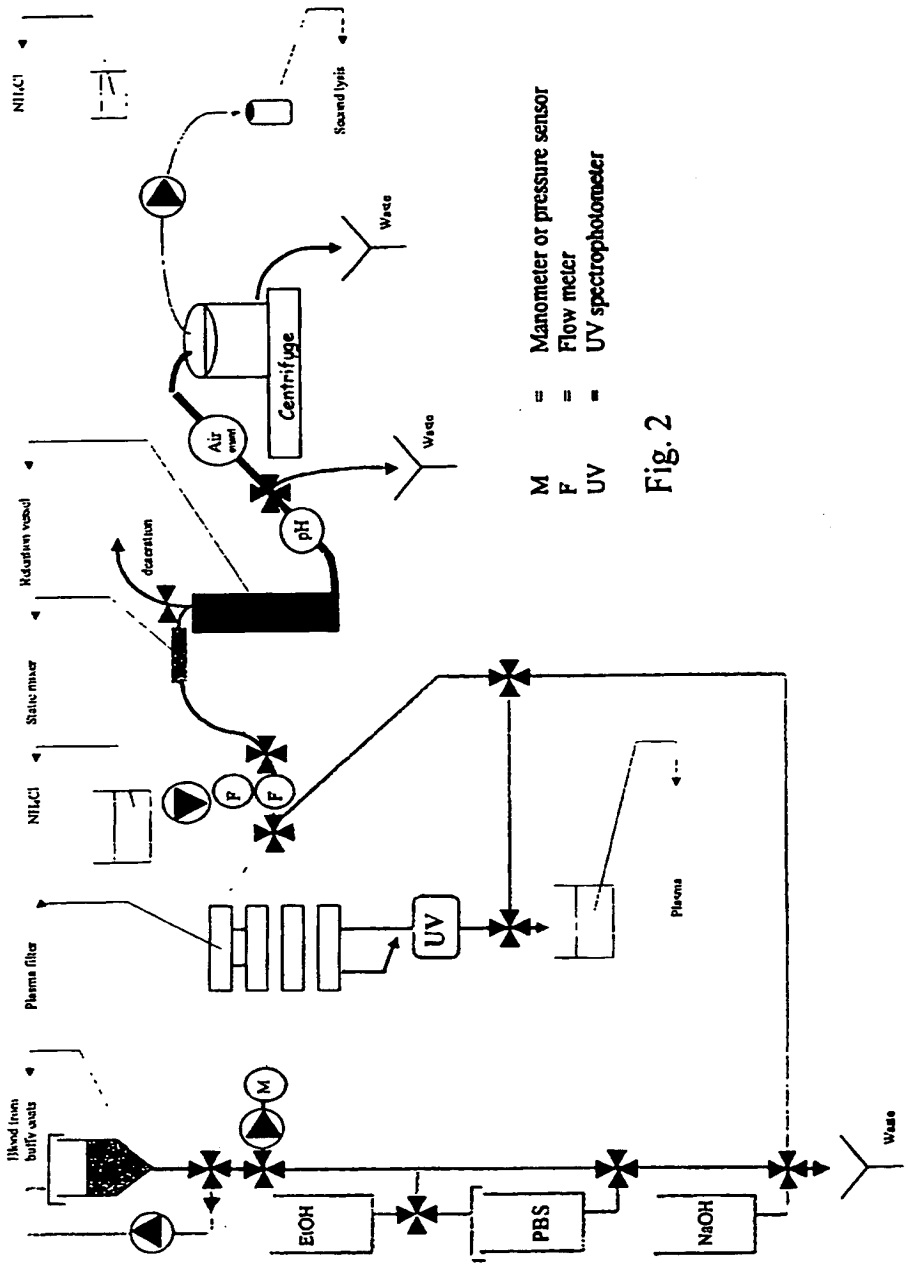
NH4Cl
Stained lysis
Waste
Centrifuge
Air vent
Waste
pH
Reaction vessel
detention
Static mixer
NH4Cl
P
F
Plasma filter
UV
Plasma
Diluted lysis buffy coats
M
EtOH
PBS
NaOH
Waste
M = Manometer or pressure sensor
F = Flow meter
UV = UV spectrophotometer

Fig. 2